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## Escape from neutralization by the respiratory syncytial virus-specific neutralizing monoclonal antibody palivizumab is driven by changes in on-rate of binding to the fusion protein

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## ABSTRACT

The role of binding kinetics in determining neutralizing potency for antiviral antibodies is poorly understood. While it is believed that increased steady-state affinity correlates positively with increased virus-neutralizing activity, the relationship between association or dissociation rate and neutralization potency is unclear. We investigated the effect of naturally-occurring antibody resistance mutations in the RSV F protein on the kinetics of binding to palivizumab. Escape from palivizumab-mediated neutralization of RSV occurred with reduced association rate ( $K_{on}$ ) for binding to RSV F protein, while alteration of dissociation rate ( $K_{off}$ ) did not significantly affect neutralizing activity. Interestingly, linkage of reduced  $K_{on}$  with reduced potency mirrored the effect of increased  $K_{on}$  found in a high-affinity enhanced potency palivizumab variant (motavizumab). These data suggest that association rate is the dominant factor driving neutralization potency for antibodies to RSV F protein antigenic site A and determines the potency of antibody somatic variants or efficiency of escape of viral glycoprotein variants.

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## Introduction

Respiratory syncytial virus (RSV) is a single-stranded, negative-sense RNA virus that is in the genus *Pneumovirus* of the family *Paramyxoviridae*. RSV is a major pediatric pathogen that causes an estimated 33 million cases of acute lower respiratory infections (ALRI) in children worldwide per year (Nair et al., 2010). RSV is also a significant pathogen among elderly and immunocompromised populations (Falsey et al., 2005). The associated respiratory disease is typically most serious during primary infection early in life, but reinfection is common throughout life (Henderson et al., 1979). Certain infants are at particular risk due to prematurity and underlying

cardiopulmonary disease, but prophylactic administration of the high-affinity RSV F-specific humanized murine monoclonal antibody (mAb) palivizumab to at-risk pediatric populations is relatively effective at preventing ALRI (Anon, 1998; Groothuis and Nishida, 2002; Top et al., 2000). Palivizumab is the only mAb for prevention of viral disease licensed to date.

One of the concerns about wide-spread clinical use of a mAb to prevent or treat infection with an RNA virus like RSV possessing an error-prone RNA polymerase is that antibody-resistant mutant viruses might emerge. Palivizumab-resistant viruses have been found in circulation in the general population (Zhu et al., 2012). It is not clear whether or not selection of antibody-resistant mutant viruses for palivizumab occurs commonly in patients or not. Some resistant strains have been observed in patients receiving palivizumab (Zhu et al., 2011), although other similar studies did not detect antibody-resistant viruses in patients on treatment (DeVincenzo et al., 2004). Predictably, it is not difficult to isolate palivizumab-resistant viruses following growth of virus in cell culture in the presence of antibody (Adams et al., 2010; Zhao et al., 2004, 2004;

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Zhu et al., 2011). Most palivizumab-resistant viruses, however, are susceptible to an affinity-matured version of palivizumab called motavizumab (Zhu et al., 2011). Motavizumab was developed as a somatic variant of palivizumab by synthetic means using engineered point mutations in a palivizumab library and selection of expressed high-affinity variants based on optimized binding *in vitro*. Motavizumab binds RSV F protein with an approximate 70-fold increase in steady-state affinity and exhibits a comparable increase in *in vivo* efficacy compared with the parental antibody palivizumab (Wu et al., 2007). Interestingly, long periods of *in vitro* selection isolated variants with long dissociation rates ( $K_{off}$ ) (and thus improved steady-state  $K_D$ ) but little functional improvement, while short time period selections identified variants with improved association rates ( $K_{on}$ ) that mediated enhanced neutralizing potency (Wu et al., 2007). Other studies to optimize palivizumab binding of RSV are consistent with the association rate being the dominant factor in the ability of mAbs to neutralize RSV by interacting with the RSV F protein antigenic site A (Wu et al., 2005). We also have shown previously that a direct relationship exists between association rate and RSV-neutralizing activity of the human mAb Fab19 using germline reversion variations of that antibody to remove individual somatic mutations in the antibody that contribute to enhanced affinity and potency (Bates et al., 2013). Association rate is not necessarily the driving factor for all antibody mediated viral neutralization, however, as studies with other viral pathogens also have shown that virus inhibitory activity can be determined predominantly by the dissociation rate (Kallewaard et al., 2008; VanCott et al., 1994).

The above observations describe mechanisms of affinity maturation of neutralizing antibodies to the RSV F protein antigenic site A. Here, we investigated the opposite phenomenon – escape from antibody-mediated neutralization by variation of the viral glycoprotein sequence. We examined the effect of viral resistance mutations in the RSV F protein on kinetics of antibody binding to RSV F protein and neutralizing activity. We hypothesized that RSV escape mutant viruses evade neutralization by palivizumab principally by altering the association rate of antibody binding, the mirror image of affinity maturation of antibodies by somatic mutations that optimize association rate.

Results

Effects of antigenic site A mutations on neutralizing activity of palivizumab

Neutralization escape mutant viruses that possessed coding changes in the antigenic site A region of the F protein were collected. The effect of these mutations on the virus neutralizing activity of palivizumab was determined by plaque reduction assay (Table 1). The specific activity (concentration need to cause 60% reduction) of palivizumab against wild-type RSV strain A2 was 0.57  $\mu\text{g/mL}$ , which is similar to previously published values (Wu et al., 2005). Palivizumab activity for each of the monoclonal antibody resistant mutant (MARM) viruses was reduced. K272N and S275F had the least effect, and reduced the specific activity of palivizumab to 0.90  $\mu\text{g/mL}$ . K272Q had the greatest effect and reduced the neutralizing activity of palivizumab to 408  $\mu\text{g/mL}$ , a 716-fold reduction in activity.

Effects of antigenic site A mutations on binding of palivizumab to RSV F proteins

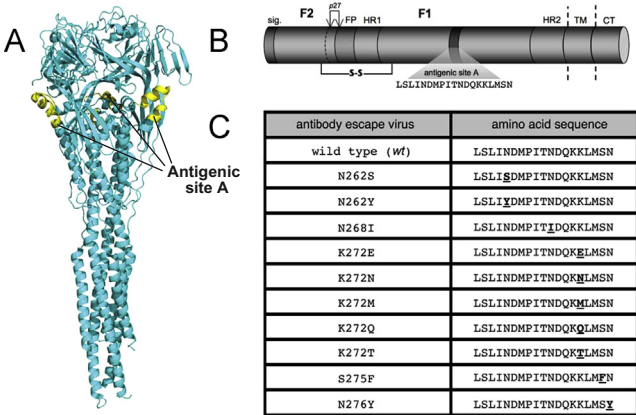
We sought to determine the effect of RSV F mutations on palivizumab binding experimentally. A number of mutations in RSV F antigenic site A that confer resistance to palivizumab have been identified previously by sequence analysis of the F gene escape mutant viruses (Adams et al., 2010; Zhao et al., 2004; Zhu et al., 2011), and a

**Table 1**  
Palivizumab-selected monoclonal antibody resistant mutant viruses exhibit greater antibody resistance *in vitro*.

MARM <sup>a</sup>	Neutralizing activity ( $\mu\text{g/mL}$ )	Fold reduction <sup>b</sup>
wt RSV A2	0.57	—
N262Y	4.8	8.4
N268I	1.1	1.9
K272E	12.5	22
K272N	0.9	1.6
K272M	2.6	4.6
K272Q	408	716
S275F	0.9	1.6

<sup>a</sup> Monoclonal antibody resistant mutant (MARM) viruses are labeled as the wild-type amino acid followed by position and then the amino acid mutation selected for by palivizumab-containing *in vitro* viral culture methods. All mutant viruses derived from the RSV wild-type strain A2.

<sup>b</sup> Fold reduction was calculated by dividing the neutralizing activity against each antibody escape mutant virus by the neutralizing activity against wild-type RSV strain A2. A higher neutralizing activity, or larger fold reduction, denotes greater viral resistance to antibody-mediated neutralization.



**Fig. 1.** The structure of RSV F and mutations that confer resistance to palivizumab. (A) Antigenic Site A is shown in gold on the previously published structure of post-fusion RSV F (PDBid: 3rrr). (B) The amino acid sequence of antigenic Site A is shown in the context of the linearized, uncleaved RSV F. (C) Specific amino acid changes within antigenic Site A which confer resistance to palivizumab are bolded and underlined.

number of these mutations also reduce binding of palivizumab to RSV F protein-expressing cells (Liu et al., 2007). We generated ten recombinant variant RSV F protein molecules, each incorporating one of the known palivizumab resistance mutations. Each of these mutations falls within the antigenic site A of RSV F protein (Fig. 1). We measured the association and dissociation rates and calculated steady-state affinity constants for palivizumab binding to each of the RSV F protein variants (Table 2). The association rates varied from a 4.9-fold decrease for K272Q to an insignificant increase of 1.2-fold for N268I and S275F relative to wild-type RSV F protein. Dissociation rates were increased for binding of palivizumab to each RSV F variant proteins. N268I had no significant effect, resulting in a 1.1-fold increase in dissociation rate, while N262Y had the greatest effect and resulted in a 28-fold increase. The N262Y mutation had a much greater effect than the S275F mutation, which resulted in a 4.2 fold increase in dissociation rate and had the second greatest effect.

Relationship of neutralizing activity to binding constants

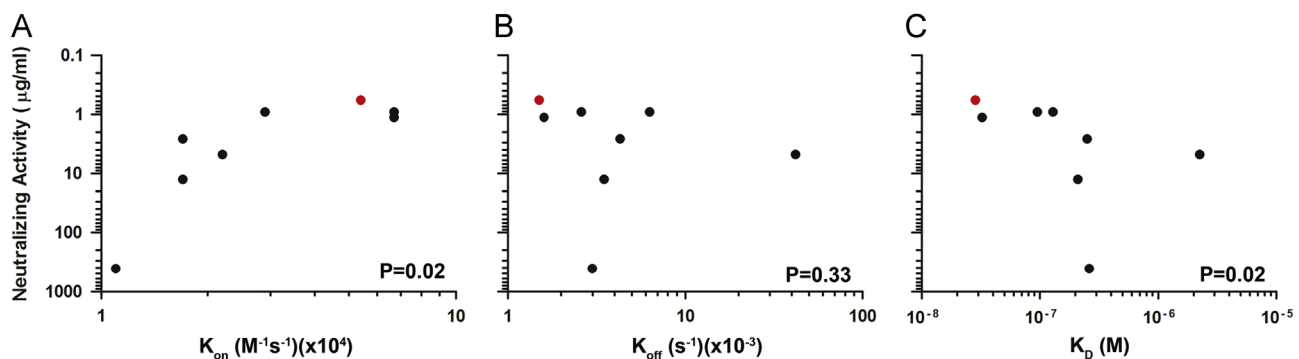
To determine the relationship of changes in binding kinetics to neutralization activity, we compared the values of  $K_{on}$ ,  $K_{off}$ , or  $K_D$

**Table 2**  
Kinetics of binding of palivizumab to RSV F protein from wild-type or antibody-resistant mutant viruses.

RSV F protein <sup>a</sup>	$K_{on}$ ( $M^{-1} s^{-1}$ ) ( $\times 10^4$ )	Fold difference <sup>b</sup>	$K_{off}$ ( $s^{-1}$ ) ( $\times 10^{-3}$ )	Fold difference <sup>b</sup>	$K_D$ (M)	Fold difference <sup>b</sup>
Wild-type	5.4	—	1.5	—	$2.8 \times 10^{-8}$	—
N262Y	2.2	−2.5	42.1	+28	$2.3 \times 10^{-6}$	−79.6
N262S	4.0	−1.4	2.8	+1.9	$8.3 \times 10^{-8}$	−2.9
N268I	6.7	+1.2	1.6	+1.1	$3.3 \times 10^{-8}$	−1.1
K272E	1.7	−3.2	3.5	+2.3	$2.1 \times 10^{-7}$	−7.4
K272N	2.9	−1.8	2.6	+1.7	$9.5 \times 10^{-8}$	−3.3
K272Y	4.0	−1.4	2.8	+1.9	$8.6 \times 10^{-8}$	−3.0
K272M	1.7	−3.2	4.3	+2.9	$2.5 \times 10^{-7}$	−8.8
K272Q	1.1	−4.9	3.0	+2.0	$2.6 \times 10^{-7}$	−9.3
S275F	6.7	+1.2	6.3	+4.2	$1.3 \times 10^{-7}$	−4.5
N276Y	1.9	−2.8	5.4	+3.6	$3.4 \times 10^{-7}$	−11.8

<sup>a</sup> Designates the wild-type amino acid followed by position and then the palivizumab-selected escape amino acid.

<sup>b</sup> Fold differences were calculated by dividing the wild-type value by the selected mutant value for fold decreases (−) or dividing the selected antibody escape mutant value by the wild-type giving fold increases (+).



**Fig. 2.** Relationship between association rate (A), dissociation rate (B), and steady-state affinity (C) of palivizumab binding to variant RSV F proteins with neutralizing activity of palivizumab against viruses bearing the same mutations. The RSV F wild type sequence is shown in red. P values indicate Spearman's Rank Correlation.

for palivizumab binding to the variant RSV F proteins with the corresponding neutralizing activity of palivizumab against each respective virus (Fig. 2). Neutralizing activity correlated with  $K_{on}$  ( $P=0.02$ ); faster association rate was associated with decreased concentration needed to neutralize. However, a significant relationship was not detected between neutralizing activity and  $K_{off}$  ( $P=0.32$ ). The relationship between  $K_{on}$  and neutralizing activity was strong enough that the  $K_D$  of palivizumab binding to variant F proteins also correlated significantly with neutralizing activity ( $P=0.02$ ).

#### Structural modeling of antigenic site A mutations at the RSV F/palivizumab interface

Structural modeling was employed to explore possible structural explanations for the observed mutational effects on binding kinetics. Based on the structure of the post-fusion RSV F protein and the structure of a high-affinity version of palivizumab (designated motavizumab) in complex with a site A peptide, we used the ROSETTA molecular modeling platform to construct a plausible structural model of the RSV F and palivizumab complex (see Methods). Motavizumab has two phenylalanines in the HCDR3 (residues 98 and 100) at the interface that are part of a hydrophobic patch (McLellan et al., 2010), yet in the structural model of palivizumab this patch is disrupted by a conformational and sequence change in the HCDR3. The structural rearrangement could be responsible for reduced binding affinity of palivizumab for RSV F. To gain a structural insight into the antibody escape mutations, computer models were generated for each mutant. The models were used to calculate the change in binding energy mediated by the escape mutations. The ROSETTA binding energies

were reasonably correlated with experimental values (Table 3) and the modeling correctly identified the escape mutation with the largest experimentally measured  $K_{off}$  and the highest  $K_D$  (N262Y; see Table 2) as the most disruptive for steady-state binding. The N262Y model suggests the bulky tyrosine side chain is bumping into the light chain of palivizumab at W53 and that a mutation to a smaller amino acid at residue 53 may resolve this clash.

#### Discussion

These studies revealed that potency of neutralization of varying strains of RSV with the licensed neutralizing palivizumab correlates directly with the association rate of the antibody binding to antigenic site A. Interestingly, mutations in the RSV F protein antigenic site A that altered the dissociation rate of this interaction did not affect the neutralizing activity of palivizumab significantly. The results provide a snapshot of the dynamic interplay between viruses and antibodies during which RNA viruses with variations in neutralizing epitopes are selected if they do not significantly reduce fitness for growth. The studies also give some specificity as to the mechanism by which some mutations facilitate escape. In this case, changes in the sequence of the viral protein that reduced the  $K_{on}$  of binding by a neutralizing antibody promoted the generation of antibody-resistant mutant viruses. These studies of RSV F antibody resistance mutations are consistent with those in previous studies of the palivizumab-F interaction showing that alterations in the antibody protein sequence that enhance  $K_{on}$ , but not changes that alter  $K_{off}$ , significantly improve neutralizing activity (Wu et al., 2005).

**Table 3**  
Comparison of experimental dG to computational binding energies.

Antigen	Exp dG	dE1 (relaxed separately)	dE2 (not relaxed separately)
WT	−10.3	45	27
N262Y	−7.7	273	236
N262S	−9.7	47	27
N268I	−10.2	111	91
K272E	−9.1	48	30
K272N	−9.6	46	27
K272Y	−9.6	61	29
K272M	−9	49	28
K272Q	−9	47	28
S275F	−9.4	74	44
N276Y	−8.8	54	28
		R=0.63	R=0.62

R values show the correlation coefficient between the experimental dG and computationally determined dE for each computational method.

The correlation between neutralization and  $K_{on}$  observed here likely reflects specific aspects of the biochemistry of infection by RSV, but the mechanism underlying the structural effect is not clear from current data. Any survival benefit conferred by mutations that reduce the  $K_{on}$  of antibody to RSV F must be balanced against the resulting effect on the usual function of F protein in the virus lifecycle. Several of the mutations studied here (K272N, K272M, K272Q, and S275F) have been examined for their effect on fusion activity of the RSV F protein (Liu et al., 2007). These mutations confer resistance to palivizumab and simultaneously enhance the fusogenic activity of RSV F (Liu et al., 2007). In the case of K272M, the fitness of the antibody resistant virus is equivalent to that of wild-type RSV strain A2 (Zhao et al., 2006). Sensitivity of virus to antibody binding with enhanced  $K_{on}$  is consistent with a mechanism of virus neutralization in which the virus is neutralized quickly and irreversibly. Recent reports suggest that the structure of the antigenic site A epitope is not transient but is preserved in the post-fusion form of RSV F, which probably occurs after attachment to cells (McLellan et al., 2011). Probably, the initial engagement of F with circulating antibodies is the limiting step in RSV neutralization mediated by antibodies to antigenic site A.

There are not enough instances of comparison of neutralization potency with association or dissociation rates to know whether  $K_{on}$  is the dominant component of binding energies driving neutralization for most viruses and epitopes or not. Clearly, there is some diversity in the factors that are needed for optimal virus inhibition. For example, we have shown that inhibition of rotavirus transcription by the human mAb RV6-26 offers a contrasting nature of the effects of binding kinetics and potency of inhibition. RV6-26 binds to a quaternary epitope of rotavirus VP6 protein in the double-layered particle at the transcription pore and prevents extrusion of RNA through the pore (Aiyegbo et al., 2013). In this antibody/virus combination, mutations that increase  $K_{off}$  result in significantly lower specific inhibitory activity (Kallewaard et al., 2008), thus the  $K_{off}$  appears to be the component of the  $K_D$  that best correlates with neutralizing activity. Because RNA extrusion likely occurs over a relatively long time compared to RSV viral fusion, the ability of the antibody to remain bound to rotavirus likely is more important than the rate at which it binds, reflecting a mechanism of inhibition in which optimized  $K_{off}$  may be of particular benefit.

There are other instances in which  $K_D$  alone does not fully inform the mechanisms underlying the biology of neutralization. Steckbeck et al. have shown that mutations in SIV gp120 that alter  $K_{on}$  and  $K_{off}$  but not the  $K_D$  can confer resistance to neutralization (Steckbeck et al., 2005). Consequently, it is conceivable that two

different antibodies could bind the same epitope with the same steady-state affinity but differences in  $K_{on}$  and  $K_{off}$  could determine significantly different profiles of neutralizing activity.

Such subtle differences in antibody-antigen binding interactions in the context of infection will be an important consideration in future structure-based vaccine development and in the development of optimized antibodies for prophylactic and therapeutic use.

## Materials and Methods

### Antibody-resistant RSV strains

RSV variant strains that were selected to be resistant to murine mAbs or palivizumab by growth and selection of escape mutant strains in the presence of antibody were described previously (Beeler and van Wyke Coelingh, 1989). We previously determined the sequences of the F protein genes for the resistant mutants, identifying critical point mutations (Crowe et al., 1998). These virus strains were isolated from the wild-type strain RSV A2. Suspensions of the antibody resistant or wild-type strains were prepared by collecting clarified supernates from HEp-2 cell monolayer cultures inoculated at 0.1 m.o.i.. Suspensions were stored at  $-80^{\circ}\text{C}$  until use in the assays.

### Measurement of palivizumab-mediated neutralizing activity against wt or antibody-resistant RSV

The neutralizing activity of palivizumab directed towards antibody-sensitive wt virus or a panel of antibody-resistant viruses was measured by a plaque reduction assay using HEp-2 monolayer cell culture. The activity was measured in  $\mu\text{g/mL}$  and calculated as the lowest concentration of palivizumab IgG needed to reduce plaque number by 60%. Diluted wt RSV strain A2 or previously characterized palivizumab-resistant RSV mutant viruses were suspended to yield 50 plaques per well and were mixed with 1:4 dilutions of palivizumab IgG, and incubated at  $37^{\circ}\text{C}$  for 60 min. Cell monolayers in 24-well tissue culture plates at 80% confluency were inoculated in duplicate by replacing the medium in each well with 100  $\mu\text{L}$  of virus-antibody mixture. After incubation at  $37^{\circ}\text{C}$  for 1 h, cell monolayers then were overlaid with 0.75% methylcellulose in Opti-MEM I (Invitrogen) supplemented with 2% FBS, 320  $\mu\text{g/mL}$  l-glutamine, 2.7  $\mu\text{g/mL}$  amphotericin B, and 45  $\mu\text{g/mL}$  gentamicin. Cultures were incubated for 5 days at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  after which the overlay was removed and the monolayers were fixed in 80% cold methanol. Plaques were stained and quantified by an immunoperoxidase staining procedure as described previously (Murphy et al., 1990). Plaques for each antibody concentration were counted, triplicate values averaged, and antibody concentration versus plaque number plotted. The 60% plaque reduction neutralizing activity ( $\mu\text{g/mL}$ ) was determined by regression curve analysis.

### Computational methods

The interactions of wt and mutant RSV F proteins with palivizumab were modeled using the ROSETTA simulation platform (Leaver-Fay et al., 2011). The complex of palivizumab and RSV F has not yet been crystallized. To make a model of the palivizumab/RSV F complex, a homology model was constructed based on the crystal structure (PDBid: 3ixt) of the motavizumab antibody in complex with a peptide corresponding to the motavizumab/palivizumab epitope on RSV F (McLellan et al., 2010). First, we expanded the motavizumab crystal structure to include the full RSV F protein by structurally aligning and then replacing the



peptide from 3ixt with the RSV F post-fusion trimer structure (PDBid: 3rrr) (McLellan et al., 2011). Second, we built a model of palivizumab by modeling the amino acids from the palivizumab sequence onto the motavizumab crystal structure. However, the structure alignment of the unbound palivizumab crystal structure (2hwz) onto motavizumab highlighted a difference in the HCDR3 conformations. Thus, the motavizumab HCDR3 was replaced by the palivizumab HCDR3 in the homology model, and a flexible backbone modeling protocol in ROSETTA was used to alleviate clashes to the HCDR3. The final palivizumab/RSV F was subjected to a constrained relax procedure. Each RSV F mutant was created using the standard fixed-backbone and side-chain minimization procedure. All the models were scored as a complex and unbound components, and then the binding score was computed with the standard ROSETTA score12 score function.

#### Construction of recombinant soluble ectodomain RSV F protein expression vectors

The wild-type (wt) RSV F ectodomain construct (pcDNA3.1- $F_{\text{ECTO-myc/His}}$ ), was constructed by PCR amplification of the ectodomain, removing the transmembrane domain and cytoplasmic tail regions from full-length RSV  $F_{\text{opt}}$  cDNA (domains and construct previously described) (Brock et al., 2005). The PCR product was cloned directionally into pcDNA3.1/myc-His B (Invitrogen) using restriction sites 5'-BamHI-EcoRI-3'. Ligated product was transformed into *E. coli* strain DH5 $\alpha$  competent cells, and plasmids were purified with the QIAprep Miniprep Kit (Qiagen). Antibody-resistant forms of RSV F protein were generated by introducing known amino acid changes from the F gene sequence of palivizumab-resistant viruses (see Fig. 1) into our F construct using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), per the manufacturer's protocol. All plasmid constructs were sequenced to confirm in-frame cloning with the C-terminal c-myc epitope and polyhistidine (6xHis) tag of the expression vector.

#### Protein expression and purification of wt or escape mutant RSV F recombinant protein

The pcDNA3.1- $F_{\text{ECTO-myc/His}}$  plasmids (wt or escape mutant forms) were transfected into 293-F suspension cells as recommended by the manufacturer (Freestyle 293 Expression System, Invitrogen). Transfected cells were incubated for 4 days, then the cells were centrifuged for 10 min at  $100 \times g$  at 4 °C and supernatant filtered through 0.2  $\mu\text{m}$  filters prior to purification and stored at 4 °C. Purification of 6xHis-tagged RSV  $F_{\text{ECTO}}$  protein was performed by immobilized metal ion affinity chromatography using pre-packed 5 mL HisTrap HP Ni-Sepharose columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's recommended protocol. Purified protein was concentrated and dialyzed against PBS through Amicon Ultra centrifugal filters with a 30 kilodalton molecular weight cutoff (Millipore, Billerica, MA). Purified preparations were identified by denaturing, non-reducing SDS-PAGE analysis as a single, silver-stained band with the expected 60 kilodalton migration. Concentration was determined by Bradford dye assay. Purified protein was stored at 4 °C until analyzed.

#### Measurement of kinetics of palivizumab IgG binding to RSV F proteins

The association and dissociation rates of wt or escape mutant RSV F recombinant protein with palivizumab IgG were measured on a Biacore 2000 biosensor (Biacore AB, Uppsala, Sweden). Palivizumab IgG (Synagis; MedImmune, Gaithersburg, MD, USA) was diluted to 30  $\mu\text{g/mL}$  in 10 mM sodium acetate, pH 4.5, and

covalently immobilized at 5  $\mu\text{L/min}$  by amine coupling to the dextran matrix of a CM5 sensor chip (Biacore AB, Uppsala, Sweden). Unreacted, active ester groups on the sensor chip were blocked with 1 M ethanolamine. For use as a reference, a blank surface, containing no palivizumab, was prepared under identical immobilization conditions. Each purified wt or antibody escape mutant RSV F protein was prepared at 5 different concentrations ranging from 5 to 500 nM in HBS/Tween-20 buffer (Biacore AB, Uppsala, Sweden), and injected over the immobilized palivizumab IgG and reference cell surfaces. Each RSV F protein, at every concentration, was run in triplicate. RSV F protein binding was measured at a flow rate of 30  $\mu\text{L/min}$  for 180 s and dissociation was monitored for an additional 360 s. Residual bound RSV F protein after each cycle was removed from the sensor chip by flowing 50 mM HCl at 100  $\mu\text{L/min}$  for 30 s. Association rates ( $K_{\text{on}}$ ) and dissociation rates ( $K_{\text{off}}$ ) were calculated by aligning the binding curves globally to fit a 2:1 binding model using BIAevaluation 4.1 software (Biacore AB, Uppsala, Sweden). Calculated rates were accepted only if the goodness of each fit based on the agreement between experimental data and the calculated fits had a  $\chi^2$  value below 5. The affinity values then were calculated for the interaction of trimeric F protein with palivizumab IgG on the chip from the equation

$$k_{\text{off}}/k_{\text{on}} = K_D.$$

We chose to use the 2:1 binding model after preliminary evaluation of the fit of binding curve using several models incorporating varying ratios of interaction revealed that the 2:1 binding model exhibited the best  $\chi^2$  values for interaction of trimeric F with bivalent IgG.

#### Statistics

Spearman's rank correlation was used to determine if changes in antibody binding kinetics among the RSV F variants had a significant impact on RSV neutralizing activity.

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